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REVIEW

What is environmental stress? Insights from fish living in a variable environment

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ABSTRACT

Although the term environmental stress is used across multiple fields in biology, the inherent ambiguity associated with its definition has caused confusion when attempting to understand organismal responses to environmental change. Here I provide a brief summary of existing definitions of the term stress, and the related concepts of homeostasis and allostasis, and attempt to unify them to develop a general framework for understanding how organisms respond to environmental stressors. I suggest that viewing stressors as environmental changes that cause reductions in performance or fitness provides the broadest and most useful conception of the phenomenon of stress. I examine this framework in the context of animals that have evolved in highly variable environments, using the Atlantic killifish, *Fundulus heteroclitus*, as a case study. Consistent with the extreme environmental variation that they experience in their salt marsh habitats, killifish have substantial capacity for both short-term resistance and long-term plasticity in the face of changing temperature, salinity and oxygenation. There is inter-population variation in the sensitivity of killifish to environmental stressors, and in their ability to acclimate, suggesting that local adaptation can shape the stress response even in organisms that are broadly tolerant and highly plastic. Whole-organism differences between populations in stressor sensitivity and phenotypic plasticity are reflected at the biochemical and molecular levels in killifish, emphasizing the integrative nature of the response to environmental stressors. Examination of this empirical example highlights the utility of using an evolutionary perspective on stressors, stress and stress responses.

KEY WORDS: Homeostasis, Allostasis, Stress, Hypoxia, Temperature, Salinity, Cortisol, Phenotypic plasticity, Acclimation, Adaptation, Intraspecific variation

Introduction

How does evolution shape the ways in which animals respond to environmental change? Do variable environments select for individuals with broad tolerance to stressors or for individuals with the ability to modify their sensitivity to stressors in response to environmental change, or both? Do highly variable environments promote or impede adaptation to local conditions? Despite substantial attention to these questions from evolutionary theorists (Auld et al., 2010; Gabriel, 2005; Gabriel and Lynch, 1992; Parsons, 2005; Via et al., 1995), answering them for any specific case remains extremely challenging, in part because the answers depend on both the temporal and spatial characteristics of the environmental variation and the characteristics of the organisms experiencing them (Angilletta and Sears, 2011). An additional complication when thinking about the evolution of responses to environmental stressors stems simply from the difficulty of clearly defining what is meant by the word 'stress'. Is there a difference between a normal homeostatic response to an environmental change and a stress response, and if so, how can we draw a dividing line between them?

Unfortunately, it is not always an easy task to decide what is meant when we use the word 'stress'. Ever since Hans Selye (Selye, 1950) first introduced the concept of stress as 'the non-specific response of the body to any demand', there have been many cogent critiques of the concept, and attempts to refine its meaning (Fink, 2009; Goldstein and Kopin, 2007; Johnstone et al., 2012; Koolhaas et al., 2011; Le Moal, 2007; McEwen and Wingfield, 2010; Romero et al., 2009). Despite these attempts, however, the definition remains ambiguous and the word is used rather differently in different contexts. Much of this confusion may stem from the fact that stress research has developed relatively independently across several fields of biology, with substantial gulfs between those interested in stress from a biomedical perspective and those interested in the effects of stressors in natural populations (Bijlsma and Loeschcke, 2005; Boonstra, 2013). Another important conceptual divide occurs between those primarily interested in the whole-organism phenomenon of the glucocorticoid-mediated stress response (Wingfield, 2013) and those interested in other aspects of responses to environmental change such as the cellular stress response (Kültz, 2005). Any general definition of the concept of stress should be able to accommodate all of these perspectives.

There is also a degree of inherent circularity in the definitions of the terms stressor, stress and stress response, and part of the confusion surrounding these concepts may arise from a lack of precision in the distinctions between them. For example, not all authors are careful to make the distinction between the word stressor and the word stress, and so stress is often used somewhat loosely to refer to both the environmental factor causing the response and the response itself. Stressors are environmental factors that cause stress. They include biotic factors such as food availability, the presence of predators, infection with pathogenic organisms or interactions with conspecifics, as well as abiotic factors such as temperature, water availability and toxicants. In addition, psychological and emotional stressors, which involve perceived threats, are extremely important in humans and other mammals (Armario et al., 2012; Campbell and Ehlert, 2012), and they are likely important in other animals as well (Galhardo and Oliveira, 2009). Organisms respond to stressors by mounting a stress response that involves a complex set of behavioural and physiological changes at multiple levels of biological organization. One important and somewhat unresolved issue in the field of stress biology is whether every environmental change that causes a response in an organism represents a stressor or whether it is possible to clearly define a level of intensity and duration of exposure that results in an environmental factor being classified as either stressful or non-stressful.

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An additional area of imprecision lies in the use of the terms stress and stress response essentially interchangeably. On the one hand, stress has been defined since the time of Selye as the response of the organism, and so these terms may be synonymous. On the other hand, I would argue that there may be some value in conceiving of stress as an intervening process between the stressor and the stress response. To further explore these ideas, here I provide a short survey of the history of concepts of stress, and then attempt to bring together the various definitions of the word stress into a single over-arching concept. Finally, I apply this framework to a case study of a fish that lives in an extremely variable environment to demonstrate the utility of this perspective on environmental stress for understanding how organisms respond to environmental change.

Stress as a threat to homeostasis

The field of stress biology originated with Selye's conception of stress as a non-specific response to changes in demand, emerging from the earlier work of Walter Cannon defining the fight-or-flight response (reviewed in Fink, 2009). Selye's definition of stress has often been restated as 'the body's response to any actual or threatened disturbance of homeostasis' (Johnson et al., 1992). However, this definition results in a concept of stress that is so broad as to be of limited utility, as stress could presumably include every transient physiological adjustment to any environmental change. If one were to apply this definition without qualification, most activities of an organism's daily life could, in principle, cause stress. In fact, Selye drew a clear distinction between individual homeostatic responses of single physiological systems and what he termed the 'general adaptation syndrome' (GAS), which he conceived of as a non-specific multisystem response to stressors (Fink, 2009; Selye, 1950). However, the GAS has been difficult to define in practice, support for its existence is mixed at best (Goldstein and Kopin, 2007) and the field continues to struggle to draw a clear distinction between normal homeostatic responses and stress responses.

Attempts to narrow the definition of stress usually involve considering only a subset of more extreme environmental factors to be stressful [e.g. 'in addition to those imposed by the normal life cycle' (McEwen and Wingfield, 2010)], or have considered only broad responses to serious challenges as meeting the definition of stress. This has led to attempts to redefine stress as 'the body's multi-system response to any challenge that overwhelms, or is likely to overwhelm, selective homeostatic response mechanisms' (Day, 2005). The difficulty with these types of definitions, of course, is to identify the type, level and intensity of environmental factors that causes stress, as opposed to a normal homeostatic response, as there may not be a sharp threshold between these two conditions.

Other approaches to refining the definition of stress have focused on whether the idea of defending homeostasis fully captures the organismal response to a stressor. In particular, these discussions have focused on the dynamic and changing nature of regulatory mechanisms. For example, Selye coined the term heterostasis to describe the process of achieving a new equilibrium state following exposure to a stressor (Fink, 2009). The term rheostasis was introduced to emphasize that the set points for homeostatic regulation may vary across environments or seasons (Mrosovsky, 1990), and the term enantiostasis was coined to refer to a situation in which multiple physiological variables are varied in order to maintain the overall functionality of a system (Mangum and Towle, 1977). These concepts emphasize the idea that maintaining functional homeostasis may require dynamic changes in a variety of parameters.

Among these various reshapings of the idea of homeostasis, the concept of allostasis (Sterling and Eyer, 1988) has received the greatest attention (McEwen and Stellar, 1993; McEwen and Wingfield, 2003). Allostasis is 'the process of achieving stability through change' (McEwen and Wingfield, 2010). Although different proponents of allostasis have used the term slightly differently and the definition has been refined by various authors (McEwen and Wingfield, 2003; McEwen and Wingfield, 2010; Sterling, 2012), in all cases, allostasis emphasizes the dynamic behavioural and physiological mechanisms that are used to anticipate or cope with environmental change to maintain organismal function.

A number of authors have reasonably questioned whether introducing the term allostasis, or indeed any of these other terms, is necessary or useful, and have instead argued that homeostasis, as conceived broadly, already encompasses all of these concepts (Dallman, 2003; Day, 2005; Romero et al., 2009). However, the various attempts at a refinement of the concept of homeostasis are important in that they point out the dynamic aspects of regulatory mechanisms that must be considered when attempting to define stress, since an environmental change that is stressful in one context may not be stressful in another.

Another important idea emerging from the discussions of allostasis is the related concept of 'allostatic load', which is defined as the wear and tear associated with the chronic overactivity or dysregulation of allostatic mechanisms (McEwen and Stellar, 1993). 'Allostatic overload' is the point at which an animal starts to suffer serious negative physiological consequences as a result of the activation of allostatic mechanisms (McEwen and Wingfield, 2010). Type I allostatic overload occurs when the energy demand from allostasis exceeds the available energy supply, resulting in reduced performance. Type II allostatic overload occurs when the prolonged activation of the allostatic mechanisms cause pathology, even when sufficient energy is available. Some have questioned whether type II allostasic overload ever occurs in nature or whether it is strictly a laboratory phenomenon (Boonstra, 2013), but putting these specific issues aside, the concept of allostatic load focuses us on the key issue of the costs of mounting a response to an environmental change, and the importance of distinguishing between responses that are beneficial, those that may impose a significant cost and those that may actually cause harm.

Stress as a hormonally mediated response

Much of the field of stress biology has focused on neurohormonal responses to stressors. In vertebrates, exposure to environmental and psychological stressors results in the activation of the sympathetic nervous system, the release of catecholamines (adrenaline and noradrenaline) and the activation of the hypothalamic–pituitary–adrenal axis (or, in fishes, the hypothalamic–pituitary–interrenal axis), which causes the release of the steroid glucocorticoid hormones (Moyes and Schulte, 2007). Invertebrates use analogous systems for responding to environmental challenges. For example, insects respond to the threat of predators by releasing the signaling molecule octopamine (Adamo and Baker, 2011), which is involved in the fight-or-flight response and is chemically similar to noradrenaline. Subsequently, a peptide hormone called adipokinetic hormone is released, which mobilizes lipid reserves to fuel the fight-or-flight response, which is analogous to the function of the glucocorticoid hormones in mobilizing glucose (Adamo, 2008; Adamo and Baker, 2011). Thus, although the specific hormones involved in the stress response differ, the hormonal response to stressors shares many conserved features across taxa.

The focus of the field of stress biology on these hormonal mechanisms has been such that glucocorticoid release is often treated as the key indicator of the occurrence of stress in vertebrates (Armario et al., 2012; Campbell and Ehlert, 2012), and the term 'stress response' is often treated as synonymous with the glucocorticoid-mediated stress response. While this greatly simplifies the detection of stress, because these hormones are easily quantified, it is not clear that this definition of stress is sufficiently broad as to encompass all situations. For example, glucocorticoid levels vary with time of day and season in many animals (Johnstone et al., 2012), but these changes may not be the result of stress. In addition, it seems possible to envisage environmental changes that are deleterious to vertebrates but that do not induce a glucocorticoid-mediated response. For example, environmental hypoxia does not always result in a release of cortisol in fish (O'Connor et al., 2011), even when it causes reductions in performance. I would argue that a deleterious environmental change such as this could reasonably be considered to be a stressor, despite the lack of a glucocorticoid hormonal stress response. Certainly unicellular organisms also have the capacity to respond to environmental challenges, and these responses are considered to be stress responses (Gasch, 2003), arguing for the utility of a broader definition of the term stress. This broader perspective would view the glucocorticoid-mediated response as simply one facet of the complete response of an organism to environmental change, which encompasses the diversity of responses at multiple levels of biological organization from the molecule to the individual to the population.

Stress as the response to unpredictable and uncontrollable environmental changes

Broadening the definition of the word stress to accommodate responses other than glucocorticoid release does not, however, provide any additional insight into the appropriate way to draw a distinction between benign and stressful environments or to clearly distinguish between normal homeostatic responses and stress responses. A number of attempts to make these distinctions have focused on the degree to which an environmental change can or cannot be anticipated or compensated for by the organism. This has led to the suggestion that 'the term stress should be restricted to conditions where an environmental demand exceeds the natural regulatory capacity of an organism, in particular situations that include unpredictability and uncontrollability' (Koolhaas et al., 2011). However, using this definition to identify stressful levels of an environmental factor might be quite difficult in practice, particularly for animals that are adapted to live in a highly variable environment, where unpredictability and uncontrollability are normal features of daily life.

Stress as reduction in fitness

Evolutionary biologists have focused on the detrimental effects of stressors by considering a stressor to be 'an environmental condition that, when first applied, impairs Darwinian fitness' (Sibly and Calow, 1989) or 'an environmental factor causing change in the biological system which is potentially injurious' (Hoffmann and Parsons, 1991). While these evolutionary definitions have a number of attractive features, they suffer from some practical and conceptual problems. First, measuring fitness is challenging in many organisms, and therefore this definition may be somewhat difficult to apply. Second, defining a stressor as any environmental change that reduces fitness might result in the conclusion that organisms in natural settings experience stress much or most of the time, as natural conditions are seldom optimal (Hoffmann and Parsons, 1991). Thus, this definition of stress (like the physiological definition of stress first proposed by Selye) may be so broad as to be of limited utility in practice. Finally, these definitions suffer from a problem of time scale. Fitness is an integrated measure of performance over an organism's lifetime, while stressors may be transient and well compensated, or chronic and highly deleterious (or anything in between). Linking processes operating at such different time scales will always be a challenging endeavour.

The appeal of evolutionary definitions of stress, with their focus on declines in fitness, is that they provide a quantitative metric and allow a clear distinction to be drawn between the concept of stress and the concept of the stress response. In this framework, stress would be considered to be the actual or potential loss in fitness as a result of exposure to a particular stressor, and the stress response would be considered to be the suite of physiological and behavioural responses to this stress. These responses may be beneficial, in that they compensate, in whole or in part, for the potential reduction in fitness. However, even beneficial responses have the potential to involve a cost, which would then factor into the fitness consequences for the organism (i.e. they could contribute to the allostatic load). Alternatively, the response could be deleterious, resulting in an even greater decline in fitness than would have occurred in the absence of the response.

Taking an evolutionary perspective emphasizes the idea that the effect of a stressor on a species is not fixed, and may vary due to mechanisms operating at various time scales. For example, within an individual's lifetime, an organism could compensate for the effects of a stressor using various types of phenotypic plasticity. Across a few generations, epigenetic changes may allow lineages to adjust to a stressor, and across many generations evolutionary adaptation may result in genetic changes at the population level that alter a species' sensitivity to a stressor. Thus, the degree of stress experienced by an organism is a consequence of both the stressor and the organism experiencing it, because an organism's sensitivity to a particular environmental factor is shaped by its prior experience and evolutionary history (Bijlsma and Loeschcke, 2005).

Because fitness is difficult to measure in practice, it may be necessary to measure various performance characteristics as a proxy for fitness in order to detect the presence of a stressor. Performance curves display the effects of the environment on a phenotype, and thus could potentially be used to detect stress. A typical performance curve has an environmental variable on the *x*-axis and fitness, or a performance trait that may be a proxy for fitness, on the *y*-axis (Fig. 1). An environmental change would then be defined as stressful at or beyond the point that fitness or performance begins to decline. Performance curves provide a way to visualize and identify stress that is testable and quantifiable. Both natural selection and phenotypic plasticity (including acclimation, developmental plasticity and transgenerational plasticity) can shape performance curves in a variety of ways, including shifting the curve along the *x*-axis, changing the performance breadth, changing the height of the curve or any combination of these shifts (Schulte et al., 2011).

A similar conceptual model of stress has been developed (Koolhaas et al., 2011) as an extension of a time-based model called the reactive-scope model (Romero et al., 2009), but without explicit reference to the rich existing literature on performance and fitness curves from evolutionary biology. In this model, the values of an environmental factor are plotted on the *x*-axis, and the so-called 'adaptive capacity' is plotted on the *y*-axis. The adaptive capacity was considered to be the ability of an individual or species to mount an adequate response to the challenges of the environment, including 'mechanisms at the level of the brain, peripheral physiology, and

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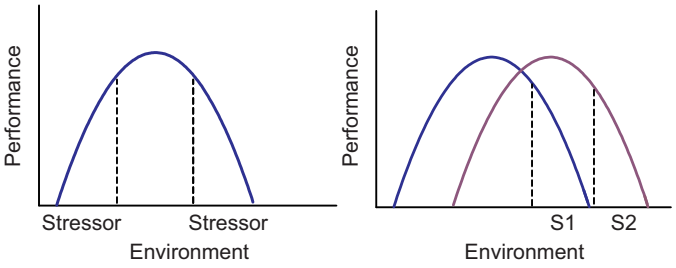

Fig. 1. Performance curves displaying the effects of an environmental variable on performance or fitness. Stressful environments are defined as those that cause performance to decline below a specified level. The left panel displays the effects of the environment on performance under the initial set of conditions. Phenotypic plasticity or adaptation can shift the performance curve of an organism (or the mean performance of a population) along the *x*-axis (right panel) such that an environment that was previously experienced as stressful (S1) no longer results in declines in performance, and instead a new range of environments (S2) provokes a stress response.

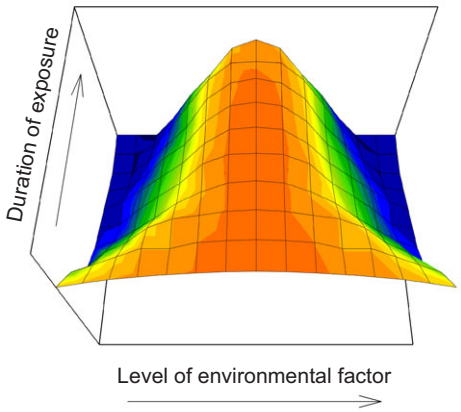

Fig. 2. Hypothetical relationship between stressor intensity, stressor duration and fitness. Fitness is highest (indicated by red) in the optimal environment, and decreases as the environment deviates from this value (indicated by blue when fitness declines to zero). Environments where fitness is lower than the optimum can be considered stressful. The extent of fitness decline and the degree of stress also depends on the duration of exposure to the stressful environments. Brief exposures may have little effect on fitness, but chronic exposure may be deleterious, and thus more stressful.

behaviour' (Koolhaas et al., 2011). The regulatory range is then the range over which adaptive capacity can be maintained. Although adaptive capacity would be difficult to measure or to quantify in practice, it is clear that the resulting curve would be analogous to a performance curve, and that the regulatory range is analogous to the performance breadth. The net result is a definition of stress (as a decline in adaptive capacity) that is essentially the same as the evolutionary definition of stress.

A unified concept of stress

Considering all of these perspectives, is it possible to develop a unified concept of stress? The evolutionary definition of stress is clearly the most broadly applicable, and it serves as an excellent starting point for a unified concept of stress. Fig. 2 illustrates a hypothetical example of how fitness (or a fitness proxy such as performance) might be affected by changes in a specific environmental variable. Both the level of the environmental variable and the length of exposure to that environment are likely to be important in determining the effect on fitness; therefore, unlike the performance curves shown in Fig. 1, this example is plotted in three-dimensional space and considers both stressor intensity and stressor duration. As the environment deviates from the optimum, fitness declines and the organism could be said to be increasingly stressed. The longer the environment deviates from its optimal condition, the greater the effect on fitness and the greater the stress.

The fitness surface shown in Fig. 2 is relatively smooth, so there is no sharp dividing line between a stressful and a non-stressful environment. However, one could also imagine a fitness surface that showed threshold effects, where fitness remains high across a range of environments and then drops precipitously once some physiological limit or threshold is reached. In this case, it might be possible to more clearly define the dividing line between a stressful and an unstressful environment.

If an organism encounters an environment that has the potential to reduce performance or fitness, it may respond by using existing phenotypic plasticity to compensate for the effects of the stressor. This phenotypic plasticity would be considered to be a stress response, and it would have the result of changing the shape of the fitness surface. For example, if the response resulted in perfect compensation, the maximum height of the fitness peak would remain the same, but its location would change, or the peak would broaden. From this perspective, an environment might be stressful for a period of time, until compensatory mechanisms return performance to its value in the original environment. Note, however, that lifetime fitness would be lower in organisms that had experienced the stressor and the resulting transient decline in performance and fitness, compared with organisms that did not experience this stressor. This description is analogous to the concept of the allostatic load in that there is a cost to experiencing the stressor, even when the stress response allows compensation. In this context, the time scale of the response is likely to be important, leading to the somewhat philosophical question of whether to call an environmental factor a stressor if the stress response is so rapid and compensation is so complete that it is not possible to measure a decline in performance or fitness at any time scale. Presumably, such a 'hidden' stressor might be revealed if the normal stress response was prevented or manipulated, with resulting detectable declines in performance or fitness during environmental challenge. Inter-individual or inter-population variability in stressor susceptibility and stress responses could also be a useful tool for detecting potential stressors in cases such as this. However, if the stress response is costly, or the compensation imperfect, the situation is much clearer: the maximum value of the fitness peak will be lower than in the optimal environment and lifetime fitness will decline for individuals exposed to the stressor, and this factor will act as a stressor at all time scales.

Although this evolutionary framework for thinking about stress has a variety of attractive features, its utility can only be assessed when it is applied to an empirical example. Here, I utilize some of the work of my research group, on the Atlantic killifish, *Fundulus heteroclitus*, to determine whether taking an evolutionary perspective on environmental stress provides any insight into the ways in which animals respond to a highly variable environment.

Killifish as an empirical example

Although the general features of responses to stressors at both the organismal and cellular levels are shared across a broad range of taxa (Kassahn et al., 2009; Kültz, 2005; Nesse and Young, 2000; Ottaviani and Franceschi, 1996), the exact nature of the environmental factors causing a stress response and the nature of the response are specific to a particular species, and indeed to

individuals within that species (Aubin-Horth et al., 2012) as a result of their evolutionary history and prior experience. Thus, stress biology is necessarily empirical, and identifies general principles based on specific examples. Here, I use Atlantic killifish (*F. heteroclitus*) as an empirical case study with which to consider the following questions: (1) can we use the evolutionary definition of stress to unambiguously identify stressors, even in an organism from a highly variable environment; (2) can intraspecific variation in stressor sensitivity evolve in such a system; and (3) what aspects of the stress response, if any, vary among individuals or populations?

I begin by briefly describing the environmental features of killifish habitats and the population genetic structure of this species, emphasizing the variable nature of the habitat and the high levels of genetic variation present in this species. I then provide a comprehensive review of the environmental factors that may act as stressors in killifish, to answer the questions outlined above.

Killifish habitat

Populations of *F. heteroclitus* are found in salt marshes along the Atlantic coast of North America. Salt marshes are highly variable environments that undergo dramatic changes in abiotic factors including temperature, salinity, oxygenation and pH at a variety of temporal and spatial scales. On a daily scale, temperature can change as much as 15°C across a single tidal cycle (Sidell et al., 1983). Similarly, dissolved oxygen levels can vary from almost complete anoxia to supersaturation (Layman et al., 2000; Smith and Able, 2004). Salinity also varies from near freshwater to full-strength seawater depending on the position in the marsh and the time in the tidal cycle (Haas et al., 2009).

In addition to daily variation, there is also pronounced seasonal variation in the abiotic characteristics of killifish habitats. For example, mean monthly temperatures vary by as much as 15°C at some locations (Schulte, 2007). In addition, the range over which temperature varies within a day differs between seasons, resulting in a shift in the maximum and minimum temperatures experienced through the year. This seasonal variation in temperature interacts with variation in other abiotic variables, producing additional seasonal patterns. For example, oxygenation of the water in marshes is affected by the photosynthetic and respiratory activity of plants, algae and bacteria. During the day, photosynthesis increases water oxygen levels, and at night respiration decreases them. Rates of both photosynthesis and respiration increase with temperature. As a result, both extreme hyperoxia and hypoxia are more common in the summer (Layman et al., 2000) and are less common in the winter.

There is also a geographical component to the environmental variation experienced by killifish, at both small and large geographical scales. At a small scale, position within a single marsh changes the range of abiotic variables encountered. For example, at the mouth of a marsh, close to the sea, salinity ranges from brackish to full-strength seawater depending on the tidal cycle and the extent of freshwater input, whereas at the upstream end of tidally influenced creeks the water may be close to fresh for much of the tidal cycle. Similarly, temperature may vary across the marsh depending on water depth, air temperature, the amount of solar radiation and the degree of influence of the tides.

Because *F. heteroclitus* is found along much of the Atlantic coast, from the St Lawrence estuary in Canada to the northern part of Florida (across a distance of over 2000 km), they are also exposed to environmental variation at much broader geographical scales. There is a steep thermal gradient along this coast, such that marshes in the northern end of the species range have, on average, a temperature more than 10°C lower than marshes at the southern end in any given month (Schulte, 2007). The temperatures at these ends of the species' range are sufficiently differentiated that in any given month, the maximum temperature experienced by a northern fish is lower than the minimum temperature experienced by a southern fish. The combination of daily, seasonal and geographical variation in temperature results in temperatures as low as −1.5°C or greater than 40°C in killifish habitats depending on the location, day and season (Layman et al., 2000; Nordlie, 2006).

Atlantic killifish do not have pelagic larvae and adults tend to remain within a relatively small area within a single marsh. In fact, the summer home range of an adult killifish has been estimated to be as low as 40 m^2 (Lotrich, 1975), although most studies suggest a home range closer to 300 m^2 (McMahon et al., 2005; Teo and Able, 2003). During the day, killifish move from tidal creeks out onto the salt marsh surface to forage but seldom move more than a kilometre, and they usually return to the tidal creeks with high site fidelity (Halpin, 2000; McMahon et al., 2005; Teo and Able, 2003). However, movements of several kilometres along tidal creeks can occur (Haas et al., 2009).

As a result of their restricted movements within a single marsh, killifish experience large fluctuations in their abiotic environment across multiple temporal and spatial scales. The high level of variation at a single location might be expected to select for individuals that are resistant to large changes in abiotic variables. In addition, the seasonal component of the environmental variation, and particularly the fact that the fish experience different ranges of environments in different seasons, might be expected to select for phenotypic plasticity. Finally, the geographic component of the environmental variation, coupled with the low dispersal potential of killifish, has the potential to result in local adaptation.

Killifish genetics

There is genetic, morphological and physiological variation among populations of *F. heteroclitus* to the point that two subspecies are recognized. *Fundulus heteroclitus macrolepidotus* occupies the northern part of the species range and is replaced by *F. heteroclitus heteroclitus* in the south, with the transition between the two subspecies centred in or just north of New Jersey (Morin and Able, 1983). This variation takes the form of a cline in gene frequencies and phenotypic traits along the coast, with different genetic markers having clines of different steepness (Adams et al., 2006; Gonzalez-Vilasenor and Powers, 1990; Powers et al., 1991; Ropson et al., 1990; Strand et al., 2012). Similar genetic and physiological clines are present in the Chesapeake and Delaware Bays, with *F. heteroclitus heteroclitus* (the southern subspecies) dominating at the coast and *F. heteroclitus macrolepidotus* dominating the freshwater reaches of these large estuaries (Powers et al., 1991).

Although the historical factors causing this distribution of genetic variation in killifish remain a matter of debate (Adams et al., 2006; Duvernell et al., 2008; Powers et al., 1991), secondary contact between two previously isolated populations following the last glaciation has likely played a role. It is possible that the original genetic differentiation between these forms was driven by environmental factors similar to those now present along the coast. Alternatively, the current distribution of genetic variation could be associated with present-day environmental variation by chance. However, for the purposes of this review, these historical arguments are less important than the fact that the killifish subspecies differ both genetically and phenotypically in a variety of traits. This variation makes them excellent models with which to examine the underlying mechanistic basis of variation in responses to environmental change.

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Stressors in killifish

Based on theoretical models of evolution in variable environments (Fitzpatrick, 2012; Gabriel, 2005; Ghalambor et al., 2007; Thibert-Plante and Hendry, 2011) one might predict that killifish would: (1) be able to cope with large changes in a range of abiotic conditions at an acute time scale, (2) be most stressed when exposed to environmental conditions (or combinations of conditions) that only occur rarely or that represent extremes at a given location or season, and (3) have the capacity to make adjustments to seasonal variation in the range of environments that they experience as stressful. In addition, given the low dispersal potential of killifish, we might also expect to observe some evidence of differentiation related to the geographic variation in conditions along the coast. Alternatively, it is possible that having to cope with a wide range of conditions across daily and seasonal scales within a single location might constrain differentiation in the ability to cope with environmental stressors across locations (Fitzpatrick, 2012; Thibert-Plante and Hendry, 2011). The next section examines the existing data on the responses of killifish to a variety of environmental stressors to determine whether it is possible to clearly differentiate between environmental factors that would be considered to be stressful versus those that would not be considered stressful, under the evolutionary definition of the term stress, and to determine whether the predictions of evolutionary models about the patterns of stress responses in killifish outlined above are fulfilled.

Behavioural and biotic stressors

There has been limited investigation of the effects of behavioural and other biotic stressors in killifish. In killifish, as in most fishes, a variety of behavioural stressors provoke a glucocorticoid-mediated stress response (Leach and Taylor, 1980). The majority of work in killifish has examined handling stress. This stressor has been shown to alter neuronal activation in the killifish brain, as demonstrated by increased expression of c-fos in various brain regions (Salierno et al., 2006), and to increase plasma cortisol, which is the primary glucocorticoid in fish (Leach and Taylor, 1980). The northern and southern subspecies of killifish differ in their susceptibility to handling stress. Fig. 3 shows the plasma levels of cortisol following a standardized handling stressor. The protocol involved either a single acute episode of handling or chronic repeated handling over the course of a week. Both stressors elevate cortisol more in southern fish than in northern fish. Interestingly, this difference may be mediated by the rate of cortisol clearance, because similar patterns occur when fish are given a slow-release cortisol implant (DeKoning et al., 2004). The subspecies also differ in the genes that are activated by cortisol release (Picard and Schulte, 2004). For example, handling stress upregulates genes encoding MAP kinases and serine threonine kinases in the livers of southern, but not in northern, fish. In addition, genetic differences between the subspecies have been detected in a glucocorticoid-responsive element within the promoter of the gene encoding lactate dehydrogenase-B (Schulte et al., 2000), and these genetic differences result in differences in the metabolic response to handling stress between the subspecies, suggesting a genetic basis for at least some of this variation. Although the consequences and adaptive significance (if any) of this variation are not known, it is clear that northern and southern killifish differ in their cortisol-mediated stress response in a variety of ways.

Salinity as a stressor

Changes in environmental salinity can be stressors in fish, but as expected, given the highly variable salinity in their environment,

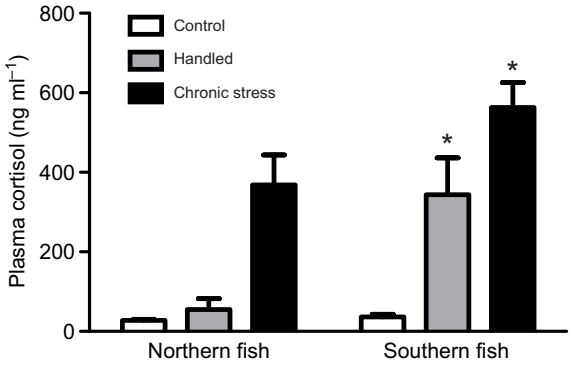

Fig. 3. Elevation in plasma cortisol following handling stress in *Fundulus heteroclitus*. The southern subspecies increases plasma cortisol levels more than the northern subspecies when exposed to a standardized handling stressor of either a single handling event (handled) or repeated handling three times daily for 7 days (chronic stress). Data are from DeKoning et al. (DeKoning et al., 2004).

killifish can tolerate salinities from near freshwater to greater than full-strength seawater (Griffith, 1974). However, the two subspecies differ in their ability to tolerate extremely low salinity, with fish of the northern subspecies able to tolerate very soft freshwater, while fish of the southern subspecies are less able to regulate plasma ion levels and experience higher mortality under these conditions (Scott et al., 2004). Neither subspecies reproduces well in freshwater, and they have poor fertilization and hatching success, but embryos of the northern subspecies can develop in lower salinities than can those of the southern subspecies (Able and Palmer, 1988). As adults, both subspecies are able to maintain ionic homeostasis down to a salinity of 0.4 ppt, but they begin to diverge at salinities below 0.1 ppt (Whitehead et al., 2012). Interestingly, clines similar to those observed along the coast are also seen in the Chesapeake and Delaware Bays, with the northern subspecies found in the freshwater end and the southern subspecies at the saltwater mouth of both bays. The observation of a repeated association between the geographic range of the subspecies that is tolerant of low salinities and the salinity of the habitats in which they are found is consistent with the action of natural selection on this trait (Whitehead et al., 2012).

The mechanisms underlying differences in the ionoregulatory ability of the adults of the two subspecies have been intensively investigated and involve differences in the ability of the gill to undergo a transition between seawater and freshwater morphologies (Scott et al., 2004; Whitehead et al., 2012). Unlike other fish, killifish maintain a seawater-type gill morphology down to salinities very close to freshwater, and make the transition to a freshwater-type morphology only if they remain in freshwater longer than a single tidal cycle (Whitehead et al., 2012), and only the northern subspecies makes a complete transition. In general, *F. heteroclitus* are found in tidally influenced parts of estuarine marshes, and they have a strong behavioural preference for brackish to full-strength seawater (Bucking et al., 2012), suggesting that they would typically use behavioural strategies to avoid freshwater in nature, but they have at least some capacity to gill remodeling if no other option is available.

Microarray studies comparing the responses of the northern and southern subspecies to low salinity suggest a role for pathways regulated by the transcription factor HNF4a (Whitehead et al., 2012) in the transition towards a freshwater-type gill morphology. In addition, there are differences in the upregulation of iodothyronine deiodinase 1 in the gill in response to freshwater challenge. This

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enzyme is responsible for the activation of thyroid hormone in peripheral tissues (Whitehead et al., 2012). This response is surprising because inhibition of the thyroid hormone axis in killifish disturbs ion regulation in seawater but not in freshwater (Knoeppel et al., 1982), and thyroid hormone levels are higher in seawater than in freshwater (McNabb and Pickford, 1970). However, thyroid hormone pathways may also play a role in freshwater adaptation in stickleback (Kitano et al., 2010), pointing to the need for a re-examination of the role of thyroid hormones in osmoregulation in freshwater fish in general (McCormick, 2001).

Together, the data on salinity tolerance at the organismal, cellular and molecular levels are consistent with the idea that only environments that are rarely experienced (i.e. extreme freshwater conditions) are highly stressful for killifish. Despite their broad salinity tolerance, the two subspecies of killifish have diverged in their ability to acclimate to freshwater, and this variation in plasticity is associated with differences in habitat choice between the subspecies, demonstrating that local adaptation in the response to an environmental stressor can occur even in species with both broad tolerance and the capacity for acclimation.

Temperature as a stressor

Killifish also tolerate large, acute changes in temperature. For example, when acclimated to 22°C, a killifish of the northern subspecies can tolerate temperatures between 1.7 and 38°C without losing equilibrium (Fangue et al., 2006). Their thermal tolerance is also extremely plastic. For example, maximum tolerated temperature is 28.5°C when a northern killifish is acclimated to 2.5°C, and 41.5°C when acclimated to 30°C (Fangue et al., 2006). Both high and low thermal tolerance differs slightly between the subspecies, with southern fish tolerating temperatures approximately 2°C higher than northern fish without losing equilibrium (Fangue et al., 2006). Both subspecies show similar plasticity in tolerance, so these tolerance differences are apparent at all acclimation temperatures above 10°C; below this temperature both subspecies are able to maintain equilibrium down to the freezing point of brackish water (−1.7°C) (Fangue et al., 2006).

Even though killifish can tolerate high (or low) temperatures acutely, these temperatures could cause stress. So exactly what temperatures are stressful for killifish? Killifish cannot be acclimated to temperatures above ~35°C without experiencing substantial mortality (Fangue et al., 2006), suggesting that long-term exposure to high temperature is extremely stressful. In addition, northern and southern killifish differ in the maximum temperature to which they can be acclimated, with northern killifish suffering 50% mortality at 36.4°C and southern killifish at 38.2°C, suggesting that the subspecies have diverged in the temperatures they experience as stressful.

Killifish acclimated to 20°C are able to maintain aerobic swimming performance with acute exposure to temperatures ranging from 15 to 25°C (Fig. 4), but performance declines beyond this range, suggesting that these temperatures may be acutely stressful (Fangue et al., 2008). Acclimation extends the thermal breadth, allowing performance to be maintained from 10 to 33°C in fish that are acclimated to their test temperature (Fig. 4). Whether this compensation of swimming performance represents true ‘beneficial acclimation’ (Berrigan and Huey, 1996; Wilson and Franklin, 2002), in the sense that this acclimation response improves fitness, remains unknown. However, even without a direct estimate of fitness, the declines in performance suggest that temperatures below 10°C and above 33°C are stressful for killifish. Patterns for the thermal sensitivity of burst (anaerobic) swimming capacity are similar, with

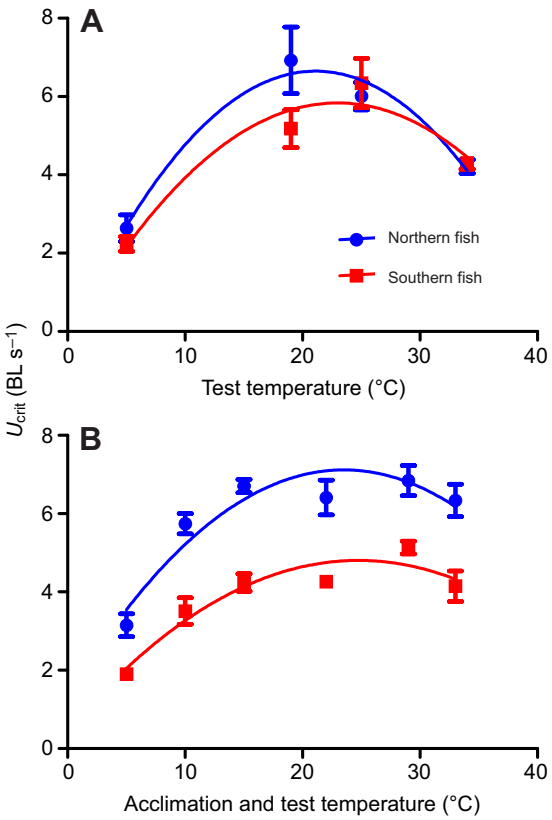

Fig. 4. Effects of temperature on swimming performance in *Fundulus heteroclitus*. (A) Aerobic swimming performance [measured as U_{crit}; mean ± s.e.m. in body lengths (BL) per second] in northern (blue line and symbols) and southern (red line and symbols) killifish acclimated to 15°C and acutely exposed to temperature change. (B) U_{crit} of both subspecies of killifish acclimated to various temperatures and tested at their acclimation temperature. Symbols as in A. Acclimation increases thermal breadth in both subspecies and significantly improves performance at extreme temperatures in the northern subspecies. Data are from Fangue et al. (Fangue et al., 2008).

fish acclimated to 10°C being able to maintain performance with acute exposure over temperatures ranging from 5 to 25°C (Johnson and Bennett, 1995). The effects of temperature on both burst performance and sustained swimming suggests that temperatures above 25°C and below 15°C may be acutely stressful to killifish acclimated to intermediate temperatures, but that physiological plasticity allows compensation to maintain performance across a broader thermal range. Thus, only temperatures above about 33°C and below 10°C would be considered to be severe stressors.

Another approach to distinguishing between stressful and non-stressful temperatures might be to examine the temperatures that killifish prefer, as organisms would be predicted to choose non-stressful environments when they are available. In fact, there is generally a strong correlation between the preferred temperature of an organism and the optimal temperature for performance (Dillon et al., 2009), and optimum performance is indicative of low stress. Fig. 5 shows the preferred temperature of killifish acclimated to 5, 15 or 25°C observed in a thermal gradient (Fangue et al., 2009). Considering the lower end of the thermal range, killifish of both subspecies avoid temperatures below approximately 12°C, which is consistent with the range of stressful temperatures defined based on swimming performance. At the high end of the thermal range, killifish generally avoid temperatures greater than 35°C. Again, these temperatures are stressful for killifish based on the effect of

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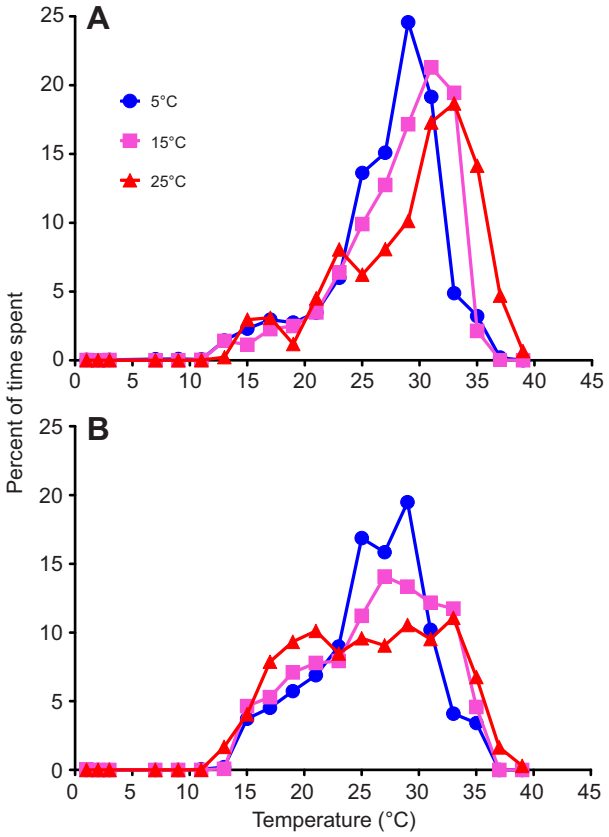

Fig. 5. Thermal preference of *Fundulus heteroclitus* acclimated to different temperatures. (A) Thermal preference of *F. heteroclitus macrolepidotus* (northern subspecies) acclimated to 5°C (blue lines and circles), 15°C (pink lines and squares) or 25°C (red lines and triangles). (B) Thermal preference of *F. heteroclitus heteroclitus* (southern subspecies). Symbols as in A. The position of each fish (*N*=9 for each subspecies) was monitored in a thermal gradient for 10 h. Data are expressed as percent of the time spent at each temperature from 14:00 to 17:00 h (to control for possible diurnal rhythms in preference) calculated in 2°C bins. Data are recalculated from Fangue et al. (Fangue et al., 2009).

temperature on swimming performance and the observation that killifish cannot be acclimated to high temperatures (36–38°C, depending on the subspecies) without substantial mortality.

In general, killifish tend to prefer temperatures at the high end of their optimal performance range, and they appear to voluntarily enter temperatures that are likely to be acutely stressful (33–36°C), based on the effects of temperature on swimming performance. This pattern is particularly obvious for northern killifish, which, depending on their acclimation temperature, show a clear preference for temperatures from 29 to 33°C, values that are at or very close to the stressful range. A preference for high temperatures is unexpected from two perspectives. First, theory suggests that organisms should prefer temperatures at the low end of their optimal range to provide a safety factor against exposure to damagingly high temperatures (Martin and Huey, 2008). Alternatively, the extreme breadth of the thermal performance curve for swimming might suggest that killifish would equally favour all temperatures within this range. So why do killifish have a strong thermal preference, and why is it so high? This pattern could be explained if performance metrics other than swimming performance are maximized at this temperature. For example, our anecdotal observations of fish in the laboratory suggest that fecundity may be maximal at ~30°C in both subspecies. However, such an argument does not explain why the preference is clearest in northern fish (and in southern fish acclimated to lower temperatures). One hypothesis consistent with this pattern is that a strong preference for high temperatures could be beneficial in situations when the reproductive period is shorter (such as in the northern part of the species' range). At higher temperatures, killifish gain the thermodynamic advantages of increased rates of reactions, which could increase growth and reproductive output. The strong preference of northern killifish for warm temperatures could allow them to take advantage of reproductive opportunities whenever they occur. This phenomenon is an example of countergradient variation (Fangue et al., 2009). Alternatively, the high thermal preference of killifish could be maladaptive. Thermal preference is thought to result from a balance of attractive and aversive inputs (i.e. killifish are attracted to warm temperatures, but avoid damagingly high ones). If the habitats of northern killifish seldom reach damagingly high temperatures, it is possible that the aversive inputs regulating preference behaviour are not strongly selected, and as a result northern killifish select higher than optimal temperatures. But exactly how risky is it for a killifish to prefer these high temperatures? Based on the data for swimming performance, only temperatures greater than 30–33°C are acutely stressful for killifish in most situations, and only temperatures even higher than this are stressful following acclimation. Temperatures above 35°C rarely occur for more than a few hours at a time in killifish habitat, even at the southern end of the species' range. Thus, a killifish may run only a slight risk of exposure to damaging temperatures if it chooses to occupy temperatures of 29–33°C. Alternatively, it is possible that the apparent thermal preference is a consequence of the way that killifish use the thermal gradient apparatus. Killifish do not spend long periods of time at these temperatures in the thermal gradient apparatus – they make brief, but frequent, excursions into them. Although they spend a substantial amount of their total time at these high temperatures, any individual exposure is generally less than a few minutes, and is usually followed by entry into much lower temperatures. The average temperature occupied by killifish in the gradient is ~27°C, which is closer to the centre of their optimal range, and several degrees below a stressful temperature. However, it is clear from the thermal preference data that killifish avoid temperatures greater than 35°C, so these temperatures are highly likely to be stressful.

Other performance traits are also consistent with the hypothesis that temperatures greater than ~33°C are acutely stressful for killifish. For example, the rate of both routine and maximum oxygen consumption increases exponentially with temperature, as would be expected based on Arrhenius effects (Healy and Schulte, 2012), but this expected pattern begins to break down with acute exposure to temperatures above 30°C in northern killifish (when acclimated to 15°C). In contrast, southern killifish maintain routine rates of oxygen consumption to 33°C, but maximum rates of oxygen consumption are slightly compromised at this temperature (Fig. 6).

These whole-organism indicators of stress are also reflected at the cellular level. For example, various heat shock proteins, which indicate a cellular stress response, are first expressed in killifish at temperatures between 30 and 33°C (Fig. 7), but with no consistent differences between the subspecies (Fangue et al., 2006). *Hsp90* is induced at lower temperatures in southern than in northern fish, whereas *Hsp70* is induced at similar temperatures. There is also no consistent pattern in differences between subspecies in the extent of induction across the genes. Microarray analysis suggests that the general features of the heat shock response are similar between the subspecies, with expression patterns indicative of increased protein

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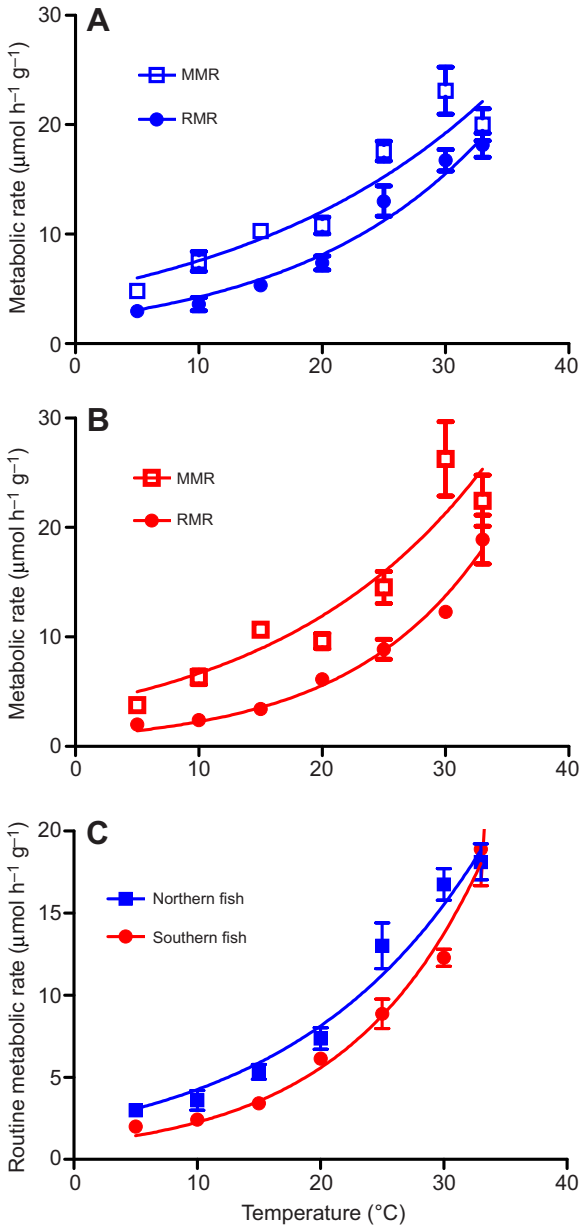

Fig. 6. Effects of temperature on metabolic rate in *Fundulus heteroclitus*. (A) Maximum metabolic rate (MMR) and routine metabolic rate (RMR) in the northern subspecies acclimated to 15°C and acutely exposed to various temperatures. (B) MMR and RMR under the same conditions for the southern subspecies. (C) Comparison of RMR for the northern and southern subspecies of *F. heteroclitus*. Data are means ± s.e.m. Lines are exponential curves fit to each data set. Note the departures from exponential expectations at temperatures above 30°C for northern fish in both RMR and MMR, and for MMR in southern fish, such that RMR converges between the subspecies at high temperatures. Data are from Healy and Schulte (Healy and Schulte, 2012).

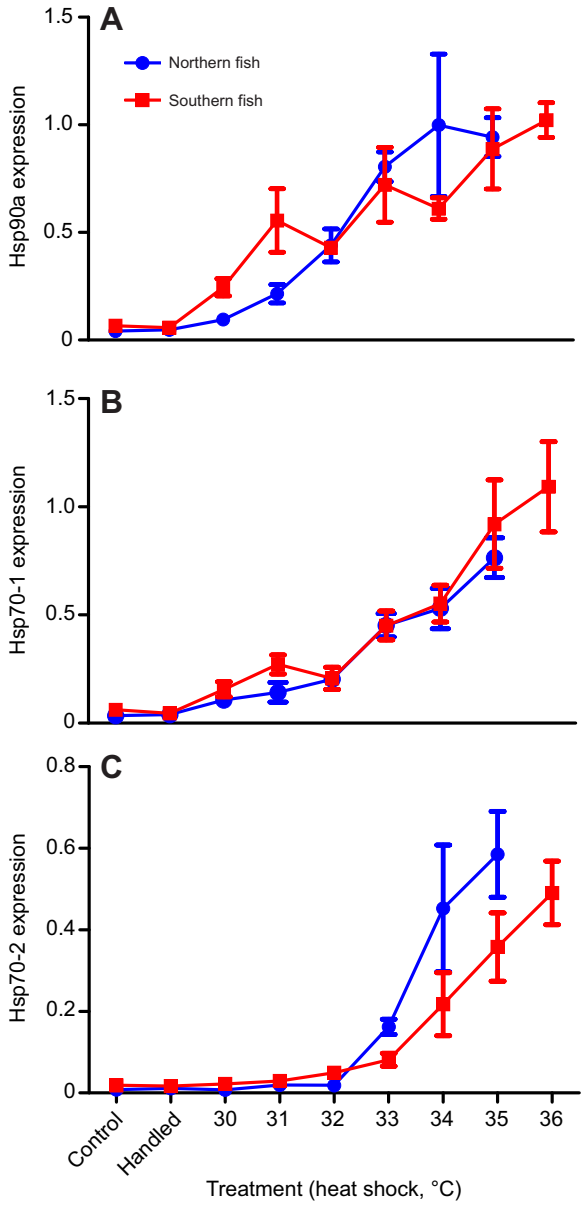

Fig. 7. Onset temperatures of heat shock protein (*hsp*) expression in *Fundulus heteroclitus*. Heat shock protein mRNA levels increase at temperatures between 30 and 33°C in northern and southern killifish (acclimated to 20°C and acutely exposed to heat shock temperatures for 2 h and sampled following 2 h of recovery). Data are relative *hsp* mRNA levels (A: *Hsp90*; B: *Hsp70-1*; C: *Hsp70-2*) normalized to the expression of elongation factor-1 alpha. Northern killifish: blue lines and symbols; southern killifish: red lines and symbols. Note that northern killifish could not be exposed abruptly to temperatures above 35°C because this caused convulsions and mortality in this subspecies in preliminary experiments. Data are means ± s.e.m. from Fangue et al. (Fangue et al., 2006).

ubiquitination, upregulation of apoptotic pathways and downregulation of global transcription (Healy et al., 2010), consistent with the patterns observed following heat shock in many species. The plasticity of the heat shock response with thermal acclimation has not yet been examined in *F. heteroclitus*. However, based on the plasticity of whole-organism thermal tolerance and performance, acclimation to higher temperatures might be expected to increase the threshold temperature for heat shock protein induction.

As is the case for whole-organism performance indicators, traits at the cellular level are consistent with the idea that that low temperatures are a severe stressor for killifish. For example, acute cold shock results in increases in circulating leukocytes because of increases in cortisol secretion (Pickford et al., 1971). Similarly, long-term exposure to cold is also stressful for killifish, as neither southern nor northern killifish are able to maintain body mass when held at 5°C in the laboratory, and thus are in negative energy balance

under these conditions (Healy and Schulte, 2012). There is some evidence that cold acclimation can modestly improve swimming performance (Fangue et al., 2008), but only in the northern subspecies. Similar patterns are evident at the cellular level, as northern killifish increase mitochondrial volume density in muscle, while southern killifish do not (Dhillon and Schulte, 2011).

Consideration of all of these data suggests that temperatures below approximately 12°C and above 30–33°C are stressful for killifish, particularly when experienced chronically. This pattern is not consistent with the hypothesis that rarely encountered environmental conditions are more stressful than more typical environmental conditions (Bijlsma and Loeschcke, 2005). Northern killifish experience temperatures between 10 and 15°C for much of the year, yet these temperatures are suboptimal for performance, and thus could be considered stressful. They also experience severe stress due to low temperatures each winter, and although there is some evidence of the evolution of compensatory responses in this subspecies, this compensation is far from perfect. Thus northern killifish must regularly experience stress even under conditions that are normal parts of their life history.

Although stress can act as a force driving adaptation (Lexer and Fay, 2005), which might be expected to result in relatively low stress in the environments most commonly occupied by a species (Bijlsma and Loeschcke, 2005), natural selection can only act if there is available genetic variation for the traits of interest, and when it is not limited by epistatic interactions or trade-offs with other traits. Natural selection does not necessarily result in a perfect match with the environment, but rather a solution that is good enough, given the circumstances. Thus, winter is stressful for northern killifish, but not so stressful that populations cannot persist.

Interacting stressors

In their complex, highly variable habitats, killifish are seldom exposed to stressors individually. Instead they must cope with multiple stressors that may interact in complex and dynamic ways. For example, we have observed that killifish acclimated to temperatures above 25°C are more likely to die following a severe handling stressor than killifish acclimated to moderate or low temperatures (T. M. Healy and P.M.S., unpublished observations). Similarly, hypoxia tolerance declines as temperature increases, but this decline is larger than would be predicted based on the effects of temperature on metabolic rate (McBryan et al., 2013). Thus, stressors may act synergistically and an environmental factor that does not represent a stressor when experienced alone could become a stressor when experienced in combination with another factor. This interaction may represent an example of the effects of allostatic load, where the costs of mounting a response to one stressor may compromise the organism's ability to cope with an additional stressor. There have been limited studies of interacting stressors in killifish [or indeed in any marine organism (Crain et al., 2008)], so the potential mechanisms causing this effect remain a fruitful area for further investigation.

Is a constant environment stressful for killifish?

From the preceding discussion, it is clear that killifish are adapted to an ever-changing, somewhat unpredictable environment. Thus, the constant environment typically imposed in the laboratory is an extremely unusual situation for a killifish. But would such an environment be stressful? There are few data available to address this question, but one data set does provide some intriguing clues. Killifish acclimated to constant conditions in the laboratory have been shown to exhibit greater levels of inter-individual variation in gene expression than fish sampled directly from the field (Scott et al., 2009), suggesting that some factor associated with the constant laboratory environment might be perceived as stressful by killifish. Alternatively, the reduction in stress imposed by holding killifish under constant near-optimal conditions might allow variation in expression otherwise masked by the responses to the stressful natural environment (Oleksiak and Crawford, 2012). In any case, it is clear that this question deserves further attention.

Conclusions

Returning to the questions that were posed at the beginning of this discussion, the case study of Atlantic killifish demonstrates the utility of taking an evolutionary perspective to define stressors, stress and stress responses. Using these definitions, we can clearly identify environments as stressful or not stressful for killifish. Having identified these environments, we can see that killifish are broadly tolerant of large changes in a wide range of environmental variables. They also show substantial plasticity in the response to stressors. However, despite their broad tolerance and plasticity, killifish often encounter stressful environments as a natural part of their life cycle (for example during winter in the northern part of the species' range). So although killifish can tolerate these conditions, and populations persist and even thrive, these conditions are stressful for this species. The differences between killifish subspecies in both tolerance and plasticity show that intraspecific variation in stressor sensitivity can evolve in organisms living in highly variable environments. The observed variation in stressor sensitivity and the stress response matches the current habitat distributions of killifish: the northern subspecies, which is more tolerant of cold temperatures and low salinities, dominates at the northern extreme of the species' range and in the freshwater reaches of major estuaries, while the southern subspecies, which is more tolerant of high temperatures and is less tolerant of freshwater, is found in the southern end of the species' range and at the saltwater mouths of the major estuaries.

At physiological and biochemical levels, these differences in stressor sensitivity and capacity for acclimation are reflected in differences in cortisol release and gene expression patterns when exposed to stressors, although these patterns are not always clear or easy to interpret. Whether this intraspecific variation in sensitivity to biotic and abiotic stressors is genetically determined, or whether it has an epigenetic component, is currently unknown. Examining the responses of killifish to environmental change shows how taking an evolutionary perspective on the definition of stress helps to clarify our understanding of the responses of organisms to environmental change, and is useful even for organisms living in extremely variable environments. This perspective may be particularly helpful as we attempt to understand the effects of the rapid, anthropogenically mediated environmental changes that organisms are currently experiencing.

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Competing interests

The author declares no competing financial interests.

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